

REAGENTS AND METHODS FOR IDENTIFYING AND MODULATING EXPRESSION OF GENES REGULATED BY RETINOIDS

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BACKGROUND OF THE INVENTION

This application claims priority to U.S. Provisional Application Serial No. 60/207,535, filed May 26, 2000.

10 This application was supported by a grant from the National Institutes of Health, No. RO1 CA62099. The government may have certain rights in this invention.

1. Field Of The Invention

15 This invention is related to changes in cellular gene expression and compounds that produce changes in cellular gene expression. In particular, the invention is related to the identification of genes the expression of which is modulated by a class of compounds known as retinoids, being chemically related to retinoic acid and Vitamin A, More specifically, the invention provides methods for identifying compounds other than retinoids that modulate expression of these cellular genes. The invention also provides reagents that are recombinant mammalian cells containing recombinant expression
20 constructs that express a reporter gene under the transcriptional control of a promoter for a gene that is regulated by retinoids, and methods for using such cells for identifying compounds other than retinoids that modulate expression of these cellular genes.

2. Summary Of The Related Art

25 Retinoids are naturally-occurring or synthetic derivatives of vitamin A. They comprise a class of clinically important differentiation agents that regulate cell growth and differentiation at the level of transcription, by binding to nuclear receptors that act as ligand-dependent transcription factors. These compounds induce cellular differentiation or terminal proliferation arrest in a number of tumor cell types *in vivo* and *in vitro* that

express retinoid receptors (Warrell, 1997, in CANCER. PRINCIPLES & PRACTICE OF ONCOLOGY (DeVita et al., eds.), pp. 483-490 (Lippincott-Raven, Philadelphia), making them useful for treating some cancers, such as acute promyelocytic leukemia and cancer chemoprevention.

5 The target of retinoid action is the cell nucleus, where retinoids bind to two types of receptors, termed RARs (retinoic acid receptors) and RXRs (retinoid X receptors) (Mangelsdorf *et al.*, 1994, "The retinoid receptors," in: The Retinoids: biology, chemistry, and medicine, Sporn *et al.*, eds., New York: Raven Press, pp. 319-351.) Retinoid-bound receptor molecules form homo- (RXR-RXR) and heterodimers (RAR-RXR) that act as transcription factors. These dimers bind to specific *cis*-regulatory sequences in the promoters of retinoid-responsive target genes, termed RARE (Retinoic Acid Response Elements), regulating their transcription. The resulting changes in gene expression are caused either directly by retinoid receptor regulation of target gene expression, or indirectly through the action of retinoid-activated signal transduction pathways, *for example*, pathways activated by the transcription factor AP-1. These gene expression changes are ultimately responsible for the growth-inhibitory effect of retinoids (Warrell, *Id.*).

10 Although retinoids have had some clinical success in cancer treatment, their use has been limited by at least two factors: development of resistance (Miller *et al.*, 1998, *Cancer* 83: 1471-1482) or toxicity. Development of resistance is due in part to alterations of retinoic acid receptors and retinoid receptor-mediated pathways (Miller *et al.*, *ibid.*). Toxicity is generally attributed to the broad physiologic consequences of retinoids, resulting from pleiotropic changes in gene expression produced by treatment with retinoids.

20 Several growth-inhibitory genes have been previously found to be inducible by retinoids in epithelial cells. None of these genes, however, was shown to be solely responsible for the growth-inhibitory effect of retinoids.

Adamo *et al.*, 1992, *Endocrinology* 131: 1858-1866 disclosed induction of insulin-like growth factor binding protein 3 (IGFBP-3) in breast carcinoma cell lines.

Swisshelm *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92: 4472-4476 identified another insulin-like growth factor binding protein, IGFBP-7 (also known as insulin-like growth factor binding protein related protein 1, or IGFBP-rP1, and mac25) as a protein induced by treatment of cells with fenretinide (4-hydroxyphenylretinamide, 4-HPR). This protein was also shown to be down-regulated in mammary carcinoma cell lines.

Kato *et al.*, 1996, *Oncogene* 12: 1361-1364 showed that introduction of mac25 cDNA into an osteosarcoma cell line inhibited growth.

Gucev *et al.*, 1996, *Cancer Res.* 56: 1545-1550 identified IGFBP-3 as a protein induced in breast carcinoma cells both by all-*trans* retinoic acid (RA) and by transforming growth factor β (TGF- β). RA-mediated growth inhibition in these cells was alleviated by introducing an antisense oligonucleotide into the cells that inhibited IGFBP-3 expression, or by introducing exogenous IGFBP-3 into the cells.

DiSepio *et al.*, 1998, *Proc. Natl. Acad. Sci. USA* 95: 14811-14815 showed that tazarotene-induced gene 3 (TIG-3, also known as retinoid-inducible gene 1, RIG-1), a putative tumor suppressor gene, is induced in primary human keratinocytes. TIG-3 shows decreased expression in cancer cells, inhibits the growth of cancer cells when expressed, and shares sequence homology with a known tumor suppressor gene, H-rev 107.

Huang *et al.*, 2000, *Molec. Cell. Endocrinol.* 159: 15-24 showed that TIG-3 was induced by retinoids in a gastric carcinoma cell line *in vitro*.

Liu *et al.*, 2000, *J. Cancer Res. Clin. Oncol.* 126: 85-90 reported that RA-induced expression of a metastasis suppressor gene, nm23-H1 in a hepatocarcinoma cell line.

5 The teachings of the prior art suggest that one mechanism of retinoid-mediated growth inhibition is the activation (or re-activation) of tumor suppressor genes and other growth-inhibitory genes that have been repressed or whose expression has been down-regulated in tumor cells. However, the reports in the art fail to indicate the identity or number of growth-inhibitory genes that are activated under the conditions of retinoid-induced growth arrest. Such reports also fail to indicate if retinoid-induced genes are
10 induced by retinoids directly, through RARE sites in their promoters, or indirectly. In the latter case, it should be possible to activate such growth-inhibitory genes even in the cells that are not responsive to retinoids.

There remains a need in this art to identify genes whose expression is modulated by retinoids, and especially growth-inhibitory genes that are induced by retinoids
15 indirectly. There is also a need in this art to develop methods for assessing the effects of compounds on expression of retinoid-modulated cellular genes, particularly growth-inhibitory genes. There is an additional need to develop alternative compounds that mimic the effects of retinoids on cellular gene expression, to which resistance is not so easily developed and that lack the toxicity and other systemic side-effects of retinoids in
20 current clinical use.

SUMMARY OF THE INVENTION

This invention provides genes whose expression is modulated by retinoids and reagents and methods for identifying compounds that mimic the effects of retinoids

without producing resistance or toxicity to said compounds.

In a first aspect, the invention provides a recombinant expression construct encoding a reporter gene operably linked to a promoter from a gene the expression of which is induced by a retinoid and which does not contain a RARE site. In preferred
5 embodiments, the reporter gene encodes firefly luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, or alkaline phosphatase.

Preferred retinoids include all-*trans* retinoic acid, fenretinide, 9-*cis* retinoic acid, 13-*cis* retinoic acid, etretinate and retinol (Vitamin A). Most preferred promoters comprising the recombinant expression constructs of the invention are promoters from a cellular
10 gene that is known to be induced by a retinoid and to have a growth-inhibitory activity.

In preferred embodiments, the cellular gene promoter is from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein FAT10 (SEQ ID NO. 4), proteasome activator PA28 subunit α
15 (PA28 α ; SEQ ID NO.:5), Mac-2 binding protein (Mac-2 BP; SEQ ID NO.:6), Protein C inhibitor (PCI; SEQ ID NO.:7), T cell receptor gamma (SEQ ID NO.:8), retinal oxidase (SEQ ID NO.:9), Bene (SEQ ID NO.:10), HIF-2 α / EPAS-1 (SEQ ID NO.:11), or selectin L (SEQ ID NO.: 12). In particularly preferred embodiments, the promoter is from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1),
20 secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein FAT10 (SEQ ID NO. 4), or proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO. 5).

In a second embodiment, the invention provides a mammalian cell containing a recombinant expression construct of the invention encoding a reporter gene under the

transcriptional control of a promoter from a retinoid-inducible cellular gene. In preferred embodiments, the reporter gene encodes firefly luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, or alkaline phosphatase.

Preferred retinoids include all-*trans* retinoic acid, fenretinide, 9-cis retinoic acid, 13-cis

5 retinoic acid, etretinate and retinol. Most preferred promoters comprising the recombinant expression constructs of the invention are promoters from a cellular gene that is known to be induced by a retinoid and to have a growth-inhibitory activity or from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in
10 neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein FAT10 (SEQ ID NO. 4), proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO.:5), Mac-2 binding protein (Mac-2 BP; SEQ ID NO.:6), Protein C inhibitor (PCI; SEQ ID NO.:7), T cell receptor gamma (SEQ ID NO.:8), retinal oxidase (SEQ ID NO.:9), Bene (SEQ ID NO.:10), HIF-2 α / EPAS-1 (SEQ ID NO.:11), or selectin L (SEQ ID NO.:12). In particularly
15 preferred embodiments, the promoter is from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein FAT10 (SEQ ID NO. 4), or proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO. 5).

20 In a third embodiment, the invention provides a method for identifying a compound that induces expression of a retinoid-inducible gene in a mammalian cell. In this method, recombinant mammalian cells according to the invention containing a recombinant expression construct of the invention encoding a reporter gene under the transcriptional control of a promoter from a retinoid-inducible cellular gene are cultured

under conditions that induce expression of at least one retinoid-induced gene in mammalian cells in the presence and absence of a compound. Reporter gene expression is compared in the cell in the presence of the compound with reporter gene expression in said cell in the absence of the compound. Compounds that induce retinoid-induced gene

5 expression are identified if reporter gene expression is higher in the presence of the compound than in the absence of the compound. In preferred embodiments, the reporter gene encodes firefly luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, or alkaline phosphatase. Preferred retinoids include all-*trans* retinoic acid, fenretinide, 9-*cis* retinoic acid, 13-*cis* retinoic acid, etretinate and retinol..

10 Most preferred promoters comprising the recombinant expression constructs of the invention are promoters of a cellular gene that is known to be induced by a retinoid and to have a growth-inhibitory activity or from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like

15 protein FAT10 (SEQ ID NO. 4), proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO.:5), Mac-2 binding protein (Mac-2 BP; SEQ ID NO.:6), Protein C inhibitor (PCI; SEQ ID NO.:7), T cell receptor gamma (SEQ ID NO.:8), retinal oxidase (SEQ ID NO.:9), Bene (SEQ ID NO.:10), HIF-2 α / EPAS-1 (SEQ ID NO.:11), or selectin L (SEQ ID NO.:12). In particularly preferred embodiments, the promoter is from human

20 insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein FAT10 (SEQ ID NO. 4), or proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO. 5). In preferred embodiments, expression of the reporter gene is detected using an immunological reagent, by hybridization to a

complementary, detectably-labeled nucleic acid, or by detecting an activity of the reporter gene product.

In a fourth embodiment, the invention provides a method for identifying a compound that induces expression of a retinoid-inducible gene in a mammalian cell. In this aspect of the invention, mammalian cells are cultured in the presence and absence of the compound. The cells are then assayed for expression of at least one cellular gene whose expression is induced by a retinoid. Compounds that induce expression of a retinoid-inducible gene in a mammalian cell are identified if expression of the cellular genes of subpart (b) is higher in the presence of the compound than in the absence of the compound. Preferred retinoids include all-*trans* retinoic acid, fenretinide, 9-*cis* retinoic acid, 13-*cis* retinoic acid, etretinate and retinol. The gene is a cellular gene that is known to be induced by a retinoid and to have a growth-inhibitory activity or human insulin-like growth factor binding protein-3 (IGFBP-3; NCBI Accession No. M35878.1), secreted cell adhesion protein β IG-H3 (Accession No. AC004503.1), epithelial protein lost in neoplasm (EPLIN; Accession No. AH009382.1), ubiquitin-like protein FAT10 (Accession No. AL031983), Mac-2 binding protein (Mac-2 BP; Accession No. U91729), Protein C inhibitor (PCI; Accession No. AL049839.3), T cell receptor gamma (Accession No. AC006033.2), retinal oxidase (Accession No. AF010260), Bene (Accession No. AP001234.3), HIF-2alpha/EPAS-1 (Accession No. NT_005065.3), selectin L (Accession No. AL021940.1), or proteasome activator PA28 subunit α (PA28 α ; Accession No. AL136295.2). In particularly preferred embodiments, the gene is human IGFBP-3, β IG-H3, EPLIN, FAT10 or PA28 α . In preferred embodiments, expression of the cellular gene is detected using an immunological reagent, by hybridization to a complementary, detectably-labeled nucleic acid, or by

detecting an activity of the gene product.

The invention also provides methods for treating an animal having cancer to prevent or ameliorate the disease. In this aspect, a therapeutically-effective dose of a compound identified by the invention that induces expression of a retinoid-induced gene is administered to an animal, most preferably a human, in need thereof.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are graphs showing the effects of retinoic acid (RA) on colony formation by MCF-7 cells. In each assay, 1.5×10^5 cells were plated per P100, in the absence or in the presence of RA. For clonogenic assays, cells were trypsinized after treatment, and 2,500 cells were plated per P100 and grown in drug-free media. Colonies comprising at least 60-80 cells were scored 12-14 days after plating; these results were normalized by the number of colonies formed by untreated cells. Each point represents the mean and standard deviation for triplicate assays. Figure 1A shows the effect of treatment with 100 nM RA for the indicated number of days. Figure 1B shows the effect of 40 hr treatment with the indicated doses of RA.

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Figures 2A through 2C show the results of RT-PCR analysis of changes in the expression of retinoid-inducible genes as described in Example 1. The identity of each gene is followed by the NCBI Accession Number (in parentheses). Figure 2A shows a time course of changes in gene expression on the indicated days after the addition of 100 nM RA. The designations "R5" and "R8" correspond to days 5 and 8 after release from

5-day RA treatment, respectively. Figure 2B shows the effects of 40 hr treatment with the indicated doses of RA. Figure 2C shows a time course of changes in gene expression on the indicated days after the addition of 1 μ M fenretinide.

Figure 3A is a photomicrograph and Figure 3B is a photograph of an immunoblot showing induction of IGFBP-3, HIF2 α /EPAS-1 and EPLIN proteins in RA-treated cells.

Figure 3A shows the results of immunocytochemical analysis of IGFBP-3, HIF2 α /EPAS-1 and EPLIN in untreated MCF-7 cells and in cells treated with 100 nM RA for 5 days. Figure 3B shows immunoblotting analysis of EPLIN in untreated MCF-7 cells and in cells treated with 100 nM RA for the indicated number of days.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides genes induced to express by retinoids. The invention also provides reagents and methods for identifying compounds that mimic the gene expression inducing properties of retinoids but lack toxicity and propensity for cells to develop resistance that is characteristic of retinoid treatment.

The present inventors have determined that retinoid treatment of retinoid-sensitive cells, such as human breast carcinoma MCF-7 cells, induces the expression of a group of genes that comprise a cohort of retinoid-inducible genes. Several of these genes are growth-inhibitory genes, *i.e.*, genes whose expression inhibits the growth or tumorigenicity of tumor cells. These genes are important, *inter alia*, as targets to be induced in tumor cells and other cells that proliferate inappropriately or pathogenically to inhibit the growth thereof. It was known in the art that genes whose expression was regulatable by retinoids contained a specific class of sequences in their promoters, termed RARE (Mangelsdorf *et al.*, 1994, in *THE RETINOIDS: BIOLOGY, CHEMISTRY, AND*

MEDICINE, (Sporn *et al.*, eds.), pp. 327-330 (Raven Press, New York). Surprisingly, however, all but one of the genes most strongly induced by retinoids in MCF-7 cells as determined by the inventors did not contain such RARE sequences in their promoters. This unexpected result indicated that retinoids activate these genes indirectly, by a mechanism that does not require RXR homodimer or RAR-RXR heterodimer binding to RARE sequences for these genes. This result also suggested that compounds other than retinoids should be capable of inducing expression of these (and perhaps other) growth-inhibitory genes in both retinoid-sensitive and retinoid-insensitive cells. Before the present invention, there was no reason to suspect that retinoid-insensitive cells could be induced to express retinoid-inducible growth inhibitory genes.

Disclosed herein is the inventors' discovery of 13 retinoid-inducible genes, including several genes having growth-inhibitory effects in mammalian cells. One of ordinary skill will appreciate that these results are not exhaustive, and other growth inhibitory genes may be induced by retinoids in mammalian cells. In view of the instant results, the skilled worker will also appreciate that some of these additional genes will be expected to lack RARE sequences in their promoters and thus be indirectly induced by retinoids. As disclosed herein, retinoid-inducible genes lacking RARE sequences in their promoters are useful targets for identifying compounds other than retinoids that mimic the physiologically-based growth inhibitory effect on cell proliferation. Identifying such compounds advantageously provides alternative agents for producing growth arrest in mammalian cells, particularly tumor cells and other cells that proliferate inappropriately or pathogenically. Such compounds are beneficial because they can mimic the growth-inhibitory effects of retinoids.

Another advantage of such compounds is that they can be expected to have a

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growth-inhibitory effect without producing systemic side effects found with other growth-inhibitory compounds known in the prior art. For example, many growth-inhibitory drugs and compounds known in the prior art disadvantageously induce p21 gene expression, which induces senescence, growth arrest and apoptosis by activating a plurality of genes, the expression of which is associated with the development of diseases, particularly age-related diseases such as Alzheimer's disease, atherosclerosis, renal disease, and arthritis (as disclosed in co-owned and co-pending U.S. Serial No. 60/_____, filed February 1, 2001 (Attorney Docket No. 99,216-E) and U.S. Serial No. 09/_____, filed May 21, 2001 (Attorney Docket No. 99,216-F), incorporated by reference herein. Retinoic acid-induced growth inhibition in MCF-7 cells, in contrast, does not induce p21 (Zhu *et al.*, 1997, *Exp. Cell Res.* 234: 293-299). The genes identified herein that are induced by retinoids are not known to be associated with any disease or disadvantageous or pathogenic effect when expressed in an animal. Thus, identification of such compounds that mimic the growth-inhibitory effects of retinoids by inducing expression of one or a plurality of the genes identified herein can be expected to have reduced or no such side-effects, making them better agents for anti-tumor and other therapies. Discovery of compounds that mimic the growth-inhibitory effects of retinoids without producing the toxic side effects of growth-inhibitory compounds known in the art is thus advantageously provided by the invention.

As provided herein, mammalian genes responsive to retinoids but not containing RARE sites in their promoters include human insulin-like growth factor binding protein-3 (IGFBP-3; NCBI Accession No. M35878.1), secreted cell adhesion protein β IG-H3 (Accession No. AC004503.1), epithelial protein lost in neoplasm (EPLIN; Accession No. AH009382.1), ubiquitin-like protein FAT10 (Accession No. AL031983), Mac-2

binding protein (Mac-2 BP; Accession No. U91729), protein C inhibitor (PCI; Accession No. AL049839.3), T cell receptor gamma (Accession No. AC006033.2), retinal oxidase (Accession No. AF010260), Bene (Accession No. AP001234.3), HIF-2alpha/EPAS-1 (Accession No. NT_005065.3), selectin L (Accession No. AL021940.1),
5 or proteasome activator PA28 subunit α (PA28 α ; Accession No. AL136295.2).

For the purposes of this invention, reference to "a cell" or "cells" is intended to be equivalent, and particularly encompasses *in vitro* cultures of mammalian cells grown and maintained as known in the art.

For the purposes of this invention, reference to "cellular genes" in the plural is
10 intended to encompass a single gene as well as two or more genes. It will also be understood by those with skill in the art that effects of modulation of cellular gene expression, or reporter constructs under the transcriptional control of promoters derived from cellular genes, can be detected in a first gene and then the effect replicated by testing a second or any number of additional genes or reporter gene constructs.
15 Alternatively, expression of two or more genes or reporter gene constructs can be assayed simultaneously within the scope of this invention.

As used herein, the term "RARE site" is intended to encompass two hexameric core motifs (as defined in Mangelsdorf *et al.*, 1994, in THE RETINOIDS: BIOLOGY, CHEMISTRY, AND MEDICINE, (Sporn *et al.*, eds.), pp. 327-330 (Raven Press, New York),
20 separated by one, two or five nucleotides), wherein the hexameric motifs are arranged as direct, inverted or palindromic repeats.

The instant inventors have shown that treatment of MCF-7 human breast carcinoma cells with low doses of retinoids induces gradual growth arrest with minimal cytotoxicity and phenotypic features of cell senescence (Chang *et al.*, 1999, *Cancer Res.*

59: 3761-3767). Relatively low doses of RA were found to induce irreversible growth arrest in MCF-7 cells, while producing only a minor reduction in cell numbers caused by cell death. This effect with "low dose" RA (between 10-100 nM) required 4-6 days of continuous exposure to RA to become apparent. Low-dose retinoid treatment was accompanied by the development of phenotypic changes in the treated cells characteristic for cellular senescence, including the development of an enlarged, flattened cellular morphology and expression of the senescence-associated marker, SA- β -galactosidase (SA- β -gal). Induction of SA- β -gal was also observed in xenografts of MCF-10T neo mammary epithelial cells *in vivo* after treatment with fenretinide. These results suggested that retinoid treatment induces senescence in tumor cells *in vivo* and *in vitro* when administered in cytostatic doses.

Senescence can be induced in a mammalian cell in a number of ways known to those with skill in the art. For example, senescence is a natural consequence of normal cell growth, either *in vivo* or *in vitro*: there are a limited number of cell divisions, passages or generations that a normal cell can undergo before it becomes senescent. The precise number varies with cell type and species of origin (Hayflick & Moorhead, 1961, *Exp. Cell Res.* 25: 585-621). Senescence can also be induced in both normal and tumor cells by treatment with cytotoxic drugs such as most anticancer drugs or radiation. See, Chang *et al.*, 1999, *Cancer Res.* 59: 3761-3767. Senescence also can be rapidly induced in any mammalian cell by transducing into that cell a tumor suppressor gene (such as p53, p21, p16 or Rb) and expressing the gene therein. See, Sugrue *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94: 9648-9653; Uhrbom *et al.*, 1997, *Oncogene* 15: 505-514; Xu *et al.*, 1997, *Oncogene* 15: 2589-2596; Vogt *et al.*, 1998, *Cell Growth Differ.* 9: 139-146. These and other means and methods for inducing senescence in mammalian cells

will be appreciated and understood by those with skill in the art, and fall within the compass of this invention.

5 The reagents of the present invention include any mammalian cell, preferably a rodent or primate cell, more preferably a mouse cell and most preferably a human cell, that can induce cellular gene expression in response to a retinoid, wherein such gene is either the endogenous gene or an exogenous gene introduced by genetic engineering. Preferred cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells.

10 Recombinant expression constructs can be introduced into appropriate mammalian cells as understood by those with skill in the art. Preferred embodiments of said constructs are produced in transmissible vectors, more preferably viral vectors and most preferably retrovirus vectors, adenovirus vectors, adeno-associated virus vectors, and vaccinia virus vectors, as known in the art. See, generally, MAMMALIAN CELL BIOTECHNOLOGY: A PRACTICAL APPROACH, (Butler, ed.), Oxford University Press: New York, 1991, pp. 57-84.

15 The invention also provides recombinant expression constructs wherein a reporter gene is under the transcriptional control of a promoter of a gene whose expression is induced by a retinoid. In preferred embodiments of this aspect of the invention, the retinoid is all-*trans* retinoic acid, fenretinide, 9-*cis* retinoic acid, 13-*cis* retinoic acid, etretinate or retinol. In preferred embodiments, the promoters are derived from genes whose expression is induced or otherwise increased by treatment of the cell with a retinoid, and preferably from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein

FAT10 (SEQ ID NO. 4), proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO.:5), Mac-2 binding protein (Mac-2 BP; SEQ ID NO.:6), Protein C inhibitor (PCI; SEQ ID NO.:7), T cell receptor gamma (SEQ ID NO.:8), retinal oxidase (SEQ ID NO.:9), Bene (SEQ ID NO.:10), HIF-2 α / EPAS-1 (SEQ ID NO.:11), or selectin L (SEQ ID NO.:12). Most preferably, the promoter is derived from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein FAT10 (SEQ ID NO. 4), or proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO.:5), Mac-2 binding protein (Mac-2 BP; SEQ ID NO.:6), Protein C inhibitor (PCI; SEQ ID NO.:7), T cell receptor gamma (SEQ ID NO.:8), retinal oxidase (SEQ ID NO.:9), Bene (SEQ ID NO.:10), HIF-2 α / EPAS-1 (SEQ ID NO.:11), or selectin L (SEQ ID NO.:12). Most preferably, the promoter is derived from human insulin-like growth factor binding protein-3 (IGFBP-3; SEQ ID NO. 1), secreted cell adhesion protein β IG-H3 (SEQ ID NO. 2), epithelial protein lost in neoplasm (EPLIN; SEQ ID NO. 3), ubiquitin-like protein FAT10 (SEQ ID NO. 4), or proteasome activator PA28 subunit α (PA28 α ; SEQ ID NO. 5). These reporter genes are then used as sensitive and convenient indicators of the effects of retinoid-induced gene expression, and enable compounds that mimic the effects of retinoids in mammalian cells to be easily identified. Host cells for these constructs include any mammalian cell. Reporter genes useful in the practice of this aspect of the invention include but are not limited to firefly luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, and alkaline phosphatase.

The following Examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature.

EXAMPLE 1
Analysis of Gene Expression Modulation by Treatment
With Retinoic Acid

5 Cytological and gene expression analyses were performed to determine the effects of retinoic acid treatment on MCF-7 cells in culture.

 Clonogenic assays were performed to analyze the differences in proliferative capacity in MCF-7 cells after treatment with 100nM RA for varying times. Cells were exposed to 100nM RA for 1-7 days and tested for their capacity to form colonies after
10 treatment at each time point. As shown in Figure 1A, plating efficiency, normalized to untreated control, decreased with time of cell incubation with 100nM RA. The decrease was initially rapid (from 100% to 40% plating efficiency after 2 days incubation with RA) and decreased more slowly to a final plating efficiency of about 10% at day 7. Figure 1B shows the plating efficiency of MCF-7 cells at varying concentrations of RA;
15 these results show a dose-dependent reduction in plating efficiency at all tested doses (as low as 10 nM). Significant reduction in plating efficiency was observed at concentrations much lower than conventionally used ($\geq 1\mu\text{M}$) to study the effects of retinoids on cell growth.

 These results were consistent with a reduction in cell growth and proliferative
20 capacity due to retinoid-modulated changes in cellular gene expression. To study gene expression changes, poly(A)+ RNA was isolated from untreated MCF-7 cells and from cells treated for 5 days with 100 nM RA. cDNA was prepared from these RNA populations and the cDNA hybridized with a cDNA microarray (Human UniGEM V cDNA microarray, Incyte Genomics, St. Louis, MO) that contains >7,000 cDNAs of
25 different human genes and ESTs. cDNA probe synthesis, hybridization with the

microarray and signal analyses were conducted by IncyteGenomics as a commercial service.

None of the genes in the microarray showed an increase in relative hybridization intensity over 2.5-fold or a decrease over 3-fold in RA-treated cells. Changes in RNA levels of a total of 47 genes that showed the biggest differences in microarray hybridization were tested by reverse transcription-PCR (RT-PCR) analysis. Of these, 27 genes showed 1.4-2.5 fold increase and 20 genes showed 1.7-3.0 fold decrease in 'balanced differential expression' (a measure of relative hybridization intensity).

RT-PCR analysis was carried out essentially as described (Noonan *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87: 7160-7164), using β -actin as an internal normalization standard. Sequences of RT-PCR primers and PCR conditions for thirteen genes most strongly induced by RA are as follows:

Table I. Oligonucleotide primers for performing PCR

Gene	Sense Primer (5' → 3')	Antisense Primer (5' → 3')
IGFBP-3	TTGCACAAAAGACTGCCAAG (SEQ ID NO.:14)	CATGAAGTCTGGGTGCTGTG (SEQ ID NO.:15)
Mac-2 BP	AATTCCACACTGTGCCCTTC (SEQ ID NO.:16)	GTGGAGTCTGGAAGGACTGG (SEQ ID NO.:17)
beta IG-H3	TGCGACTAGCCCTGTCTAT (SEQ ID NO.:18)	CATGCACAAAGGCTCACATCT (SEQ ID NO.:19)
PCI	GCACCCAAAGAGCAAGACTTC (SEQ ID NO.:20)	CGAGCTGCCCTCTTTTGAAC (SEQ ID NO.:21)
FAT 10	AATGCTTCCTGCCCTCTGTGT (SEQ ID NO.:22)	ATCACTGGGCTTACCCACTT (SEQ ID NO.:23)
EPLIN beta	AGAAAGGGGACCCTGACTGT (SEQ ID NO.:24)	AAGATCCTCACCGTCCTTGA (SEQ ID NO.:25)
T cell receptor gamma	AGGAGCTGTGGAAACATGG (SEQ ID NO.:26)	CATAACAGACGGTGGCACAA (SEQ ID NO.:27)
P28 alpha	ACAGGTGGATGTGTTTCGTG (SEQ ID NO.:28)	TTCATCCTCCCCCTTCTTCT (SEQ ID NO.:29)
Retinal oxidase	GTGGTGGACATCATGACAGC (SEQ ID NO.:30)	AGCGGCTCCAAAGTCTTGATA (SEQ ID NO.:31)
Bene	CCAGGCAACAAAGGAGAGA (SEQ ID NO.:32)	TGCCTTCTGTCAATTGGGAAT (SEQ ID NO.:33)
HIF-2alpha /EPAS-1	CCAGTGCATCATGTGTGTCA (SEQ ID NO.:34)	CCCGAAATCCAGAGAGATGA (SEQ ID NO.:35)
L-selectin	GTGGCACCTCCTACGTCAAA (SEQ ID NO.:36)	TGAATCCTTTCCCTTTATGGTC (SEQ ID NO.:37)
RNF	GAGGTGCAGTCCAAAGGAA (SEQ ID NO.:38)	TGTGTTGGCGTACAGGTCITTTG (SEQ ID NO.:39)
Beta-actin	TGTGTTGGCGTACAGGTCITTTG (SEQ ID NO.:40)	TGTGTTGGCGTACAGGTCITTTG (SEQ ID NO.:41)

Table II. Temperature conditions for PCR (in °C)

Gene	Denaturation	Annealing	Extension	Cycles	Product size
IGFBP-3	95	60	72	27	247
Mac-2BP	95	60	72	29	249
beta IG-H3	95	60	72	27	199
PCI	95	60	72	28	249
FAT 10	95	60	72	27	246
EPLIN beta	95	60	72	26	261
T cell receptor gamma	95	60	72	26	252
PA28 alpha	95	60	72	27	250
Retinal oxidase	95	60	72	29	197
Bene	95	60	72	26	265
HIF-2 alpha/EPAS-1	95	60	72	26	250
L-selectin	95	60	72	26	195
RNF	95	60	72	29	200
beta actin	95	60	72	21	275

RT-PCR assays confirmed altered expression for 43 of 47 genes and showed that 13 upregulated genes were induced by RA much more strongly (5-10 fold or more) than indicated by microarray hybridization; these results are shown in Figures 2A and 2B. Time course analysis of changes in RNA levels of these 13 genes (Fig. 2A) showed that many of them increased their expression between days 1 and 4 of RA treatment, in parallel with the loss of clonogenicity (shown in Fig. 1A). Analysis of RA dose-dependence of gene expression (shown in Fig. 2B) showed that almost all of these genes were induced even by the lowest (10 nM) dose of RA that produced detectable loss of clonogenicity (shown in Fig. 1B). All 13 genes were also induced by treatment with 1 μ M of another retinoid, fenretinide, which is used in breast cancer chemoprevention (these results are shown in Fig. 2C).

Induction of three genes in this group was tested and confirmed at the protein level, by immunocytochemical assays, shown in Figure 3A, using rabbit polyclonal antibody against EPLIN (a gift of Dr. David Chang, UCLA), and goat polyclonal antibodies against IGFBP-3 and EPAS-1/HIF-2 α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and standard techniques. Antibody staining was detected using Vectastain kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. These results show that induction of IGFBP-3, EPAS-1/HIF2 α , and EPLIN mRNA was accompanied by increased expression of the corresponding proteins in RA-treated cells.

These results showed that RA and fenretinide strongly induced the expression of a common set of genes under the conditions where these retinoids inhibit cell growth and induce the senescent phenotype.

EXAMPLE 2
Biological Functions of Genes Induced in MCF-7 Cells by Treatment
With Retinoic Acid

5 The genes detected as discussed in Example 1 were found by literature research to have biological functions that are relevant to the cellular effects of retinoids.

Strikingly, 4 of 13 genes that are strongly induced by retinoids have been reported to possess antiproliferative activity. The first gene encodes insulin-like growth factor binding protein-3 (IGFBP-3), a secreted factor that was shown to be inducible by RA in breast carcinoma cells and to inhibit the growth of these cells (Adamo *et al.*, 10 1992, *Endocrinology* 131: 1858-1866; Gucev *et al.*, 1996, *Cancer Res.* 56: 1545-1550). In addition to its role in IGF sequestration, IGFBP-3 was recently found to bind and modulate the transcriptional activity of a retinoid receptor RXR α (Liu *et al.*, 2000, *J. Biol. Chem.* 275: 33607-33613). Induction of IGFBP-3 was confirmed by immunocytochemical assays shown in Fig. 3A.

15 Another growth-inhibitory gene induced by treatment with retinoids encodes secreted cell adhesion protein β IG-H3, which is inducible by TGF- β in several cell types (Skonier *et al.*, 1992, *DNA Cell Biol.* 11: 511-522). Transfection of β IG-H3 was shown to inhibit the tumorigenicity of Chinese hamster ovary cells (Skonier *et al.*, 1994, *DNA Cell Biol.* 13: 571-584). β IG-H3 is expressed in normal but not in neoplastically 20 transformed human fibroblasts (Schenker & Trueb, 1998, *Exp. Cell Res.* 239: 161-168), suggesting that this gene may be a tumor suppressor.

The third gene encodes a LIM domain protein termed EPLIN, an actin-binding protein that is expressed in primary epithelial cells but downregulated in different types of carcinomas (Maul & Chang, 1999, *Oncogene* 18: 7838-7841). Ectopic expression of 25 EPLIN was shown to suppress cell proliferation in an osteosarcoma cell line (Maul &

Chang, *Id.*). The EPLIN gene encodes two protein isoforms, EPLIN α and EPLIN β , with the larger (β) isoform showing a stronger growth-inhibitory effect. The observed induction of EPLIN gene expression was confirmed by immunocytochemical and immunoblotting assays (results shown in Figures 3A and 3B). These assays were performed using rabbit polyclonal antibody against EPLIN (a gift of Dr. David Chang, UCLA) and were carried out by standard techniques. Antibody staining was detected using Vectastain kit (Vector Labs) for immunocytochemistry and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) for immunoblotting. Electrophoretic mobility of EPLIN in MCF-7 cells (110 kDa; Fig. 3B) corresponds to the β isoform, consistent with the art that showed stronger growth inhibition by this isoform.

The fourth gene encodes an ubiquitin-like protein FAT10. FAT10 interacts with a mitotic spindle protein Mad2, and its overexpression in HeLa carcinoma cells was reported to be detrimental to cell survival (Liu *et al.*, 1999, *Proc. Natl. Acad. Sci. USA* 96: 4313-4318).

Retinoic acid treatment is known to promote proteasome-mediated degradation of retinoic acid receptor RAR α (Zhu *et al.*, 1999, *Proc. Natl. Acad. Sci. USA* 96:14807-14812) and of cyclin D (Spinella *et al.*, 1999, *J. Biol. Chem.* 274: 22013-22018). Proteasome-mediated cyclin D degradation has been proposed as a mechanism for retinoid-induced growth arrest (Spinella *et al.*, *Id.*). Remarkably, one of the RA-induced genes encodes proteasome activator PA28 subunit α (PA28 α). Expression of PA28 α is sufficient to activate the proteasome (Groettrup *et al.*, 1996, *Nature* 381:166-168), and the induction of this gene may account at least in part for proteasome activation by retinoids. Pa28 α therefore can be regarded as another growth inhibitor.

Still another RA-induced gene encodes retinal oxidase (aldehyde oxidase), an enzyme that catalyses the final step of RA synthesis from vitamin A (Huang *et al.*, 1999, *Arch. Biochem. Biophys.* 364: 264-272). The observed induction of retinal oxidase suggests that retinoid treatment may stimulate RA synthesis in the treated cells, providing a potential positive feedback mechanism.

Aside from β IG-H3, two other induced genes encode secreted proteins that may contribute to the senescence-like flattened morphology and increased adhesion of RA-treated MCF-7 cells. One of these encodes Mac-2 binding protein (Mac-2 BP), a cell adhesion factor of the extracellular matrix (Sasaki *et al.*, 1998, *EMBO J.* 17: 1606-1613). Mac-2 BP is also upregulated in p21-induced senescence of human fibrosarcoma cells (Chang *et al.*, 2000, *Proc. Natl. Acad. Sci. USA* 97: 4291-4296). The other gene encodes protein C inhibitor (PCI), a non-specific serine protease inhibitor, which is normally produced by the liver. While retinoid-treated MCF-7 cells express markers of senescence, none of the genes that are strongly induced by RA in this cell line has been associated with epithelial cell differentiation. Two of the induced genes, however, encode transmembrane proteins specific for the hematopoietic lineage, including the leukocyte homing receptor L-selectin and T-cell receptor. Induction of these genes correlates with a well-documented differentiating effect of retinoids in hematopoietic malignancies (Warrell, 1997, *ibid.*). RA also induces another transmembrane protein, Bene, which has no known function.

The last two RA-induced genes encode known or putative transcriptional regulators. One of them is HIF-2 α /EPAS-1, a member of a family of PAS domain transcription factors that mediate the effects of hypoxia and some other stress factors on gene expression (Semenza, 1999, *Annu. Rev. Cell. Dev. Biol.* 15: 551-578).

Interestingly, IGFBP-3 is also one of the hypoxia-stimulated genes (Feldser *et al.*, 1999, *Cancer Res.* 59: 3915-3918). Induction of HIF-2 α /EPAS-1 was confirmed by immunocytochemical assays shown in Fig. 3A.

The final RA-induced gene encodes a ring finger protein RNF (accession number YO7828). While the RNF function is unknown, it shares 25-38% amino acid identity with a family of regulatory proteins, some of which have been implicated in retinoid response, senescence or differentiation. These include TIF1 α , which functions as a ligand-dependent transcriptional coactivator of retinoid receptors (Le Douarin *et al.*, 1995, *EMBO J.* 14: 2020-2033), as well as promyelocytic leukemia (PML) gene, which is fused with RAR α in the t(15;17) translocation in PML (Kakizuka *et al.*, 1991, *Cell* 66: 663-674). PML has been recently identified as a mediator of accelerated senescence induced by mutant RAS in human fibroblasts (Ferbeyre *et al.*, 2000, *Genes Dev.* 14: 2015-2027). Another member of the same family is HERF1, which is required for terminal differentiation of erythroid cells (Harada *et al.*, 1999, *Mol. Cell. Biol.* 19: 3808-3815). Interestingly, HERF1, RNF and FAT10 all map to the major histocompatibility locus on chromosome 6p21.3. This locus also contains the gene for RAR α , which was reported to be induced by RA (Shang *et al.*, 1999, *J. Biol. Chem.* 274:18005-18010) and to be upregulated in senescent mammary cells (Swisshelm *et al.*, 1994, *Cell Growth Differ.* 5: 133-141).

As disclosed herein, retinoid treatment of breast carcinoma cells concurrently induces several genes with known antiproliferative functions, including candidate tumor suppressors that are selectively downregulated in neoplastic cells (EPLIN and β IG-H3). Since the UniGem V array comprises only a fraction of all human genes, the actual number of growth inhibitors that are co-induced by retinoids should be much higher than

the genes identified herein. Such additional retinoid-inducible growth inhibitors can be readily identified, however, by hybridizing cDNA probes described herein with larger cDNA arrays or combinations of arrays, or by carrying differential cDNA cloning using methods that are well known in the art (*see, for example*, International Patent Application, Publication No. WO00/61751, incorporated by reference herein.

These results demonstrated that retinoids can induce several growth-inhibitory genes, which provide a basis for developing reagents for screening compounds capable of inducing one or more of these genes without producing retinoid-associated resistance or toxicity.

EXAMPLE 3
Construction of Retinoid-regulated Promoter-Reporter Gene Constructs
That Are Induced with Retinoic Acid

In order to produce reporter gene constructs under the transcriptional control of retinoid-induced genes, promoter sequences for all 13 genes that are strongly induced by retinoids, comprising 1400-1500 bp upstream of the 5' end of the longest available cDNA sequence of the respective genes, were identified in the human genome database.

These sequences were then analyzed for the presence of two closely spaced hexameric core motifs of RARE sites (Mangelsdorf *et al.*, 1994, in THE RETINOIDS: BIOLOGY, CHEMISTRY, AND MEDICINE, (Sporn *et al.*, eds.), pp. 327-330 (Raven Press, New York), in variable orientations, using Regulatory Sequence Analysis Tools, available at: <http://www.ucmb.ulb.ac.be/bioinformatics/rsa-tools/>.

A putative RARE site found in only one promoter, ring finger protein RNF, where the sequence:

AGGTCACAGCCAGTTCA

(SEQ ID No.:42)

(**boldface** indicates the RARE core motifs; Mangelsdorf *et al.*, 1994, *ibid.*) appears in inverse orientation about 360 bp upstream of the apparent transcription start site. None of the other promoters contained discernable RARE sequences, suggesting that most of these genes are induced by retinoids through indirect mechanisms. Interestingly, RNF is also the only gene in this group to reach its maximum expression after just one day of treatment (*see* Fig. 2A), suggesting that RNF is likely to be directly inducible by retinoids.

It is remarkable that none of the growth-inhibitory genes that show strong and sustained induction in RA-treated MCF-7 cells contain RARE sites in their promoters, suggesting that it may be possible to induce these growth-inhibitory genes in cells that lack retinoid receptors, and using non-retinoid inducing agents. Reporter gene-containing constructs, under the transcriptional control of a promoter from a retinoid-induced gene, particularly a gene lacking a RARE sequence in the promoter, enable screening of test compounds for the capacity to induce gene expression from these genes in a way that mimics the gene-inducing effects of retinoids without producing toxicity or development of resistance.

Such reporter gene constructs are prepared as follows. The promoter region of a retinoid-regulated gene, such as β IG-H3, is identified in the genomic sequence (NCBI accession number AC004503) as adjacent to the 5' end of the cDNA. Polymerase chain reaction (PCR) amplification of the promoter-specific DNA is performed using genomic DNA from human MCF-7 cells as the template and the following primers for β IG-H3:

5' GGCCAGGTGCCTCTTCTTAG 3' (sense) (SEQ ID NO.:43)

and

5' CGGCTCCAGGGAAGTGAG 3' (antisense) (SEQ ID NO.:44)

using *PfuTurbo* DNA Polymerase (Stratagene) and 28 cycles of PCR where each cycle consisted of 45 sec. at 95°C, 1 min 30 sec. at 60°C, and 2 min. at 72°C. A 1020 bp fragment is amplified using this method and cloned into the TOPO TA cloning vector pCRII/TOPO (Invitrogen). The sequence identity of this construct is verified, and the

5 *HindIII-Xho I* fragment containing the promoter in the correct orientation is then inserted into the *HindIII* and *Xho I* sites in a firefly luciferase-reporter vector pGL2-Basic (Promega, Madison, WI) using standard recombinant genetic techniques (Sambrook *et al.*, 1990, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press: New York).

10 The ability of this construct to drive retinoid-inducible luciferase expression in mammalian cells is demonstrated in transient transfection assays, as described in U.S. Provisional Patent Application Serial No. 60/_____, filed February 1, 2001 (Attorney Docket No. 99,216-E) and U.S. Patent Application Serial No. 09/_____, filed May 21, 2001 (Attorney Docket No. 99,216-F), incorporated by reference herein.

15 Briefly, transfection is carried out using LIPOFECTAMINE 2000 (Life Technologies, Inc. Gaithersburg). Cells are plated at a density of 70,000 cells/ well in 12 well plates in 1 mL media containing 2mM glutamine, 10% FBS, 0.1mM NEAA (Non- Essential Amino Acids , GIBCO), 1mM sodium pyruvate, and 10µg/mL insulin, and without penicillin/streptomycin. After culturing the cells for a sufficient time that they attached

20 to the culture dish, transfection was performed in triplicate according to the manufacturer's instructions, using 1 µg pGL2- basic vector DNA and 1µg pGL2- β IG-H3 promoter DNA. After 10 hours, culture media is replaced with media containing penicillin/ streptomycin at standard tissue culture concentrations. The cells were then incubated in the presence or absence of 100nM atRA for 72 hours. After incubation,

cells are washed twice with phosphate-buffered saline and collected in 100 μ L of Reporter Lysis Buffer (Promega). The lysate is left at room temperature for 10 minutes followed by 1 cycle of freeze / thaw using a dry ice-ethanol bath for freezing the cell sample and thawing in a 37°C water bath. 50 μ L aliquots are transferred to fresh tubes for Firefly Luciferase Assay (Promega). Luciferase activity is measured as described above using a Turner 20/20 luminometer at 47.9% sensitivity with a 5 sec. delay period and 15 sec. integration time. An additional aliquot is removed from the cell lysate to measure protein concentration using Bio-Rad protein assay kit (Bradford assay). Luciferase activity for each sample is normalized to protein content and expressed as luciferase activity/ μ g protein. All assays are carried out in triplicate and displayed as a mean and standard deviation.

To develop a stably transfected cell line with retinoid-regulated luciferase expression, the construct described above is introduced into a cell line that is susceptible to growth inhibition by retinoids, such as MCF7 cells by cotransfection with a vector encoding a selectable marker, such as pBabePuro, carrying puromycin N-acetyltransferase as a selectable marker. Transfection is carried out using LIPOFECTAMINE 2000 (Life Technologies, Inc., Gaithersburg, MD), using a 10:1 ratio of the construct and a plasmid or other vector containing a selectable marker. Stable transfectants are selected using an appropriate amount of a selecting agent specific for the selectable marker encoded by the plasmid or vector. Selective agent-resistant cell lines are isolated and tested for luciferase activity (using a Luciferase Assay System, Promega), in the presence and in the absence of 100 nM RA, or another retinoid at a concentration that produces growth inhibition in the recipient cell line.

This assay is performed as follows. Cells are plated at a density of 40,000

cells/well in 12 well plates in 1 mL of media containing penicillin/streptomycin, glutamine and 10% fetal calf serum (FCS). After attachment, cells are treated with 100nM RA or left untreated for different periods of time. Cells are washed twice with phosphate-buffered saline and collected in 300 μ L of Reporter Lysis Buffer (RLB; Promega). The lysate is centrifuged briefly at 10,000 g to pellet debris, and 50 μ L aliquots are transferred to fresh tubes for use in the Firefly Luciferase assay (Promega). Luciferase activity is measured using a Turner 20/20 luminometer at 55.6% sensitivity with a 5 second delay period and 15 second integration time. An additional aliquot is removed from the cell lysate to measure protein concentration using the Bio-Rad protein assay kit (Bradford assay). Luciferase activity for each sample is normalized to protein content and expressed as luciferase activity/ μ g protein. All assays are carried out in triplicate and displayed as a mean and standard deviation.

Such constructs and cells provide a basis for a screening assay for identifying compounds that induce retinoid-induced gene expression. The same type of screening can also be conducted using transient transfection assays with promoter constructs of retinoid-inducible genes rather than stably-transfected cell lines. The methods for high-throughput screening based on luciferase expression are well known in the art (see Storz *et al.*, 1999, *Analyt. Biochem.* 276: 97-104 for a recent example of a transient transfection-based assay and Roos *et al.*, 2000, *Virology* 273: 307-315 for an example of screening based on a stably transfected cell line). Compounds identified using these cells and assays are in turn useful for developing therapeutic agents that can induce gene expression of retinoid-inducible genes without the concomitant toxicity or tendency to produce retinoid resistance.

The absence of retinoid responsive elements in the promoters of almost all of the

genes shown herein to be induced by retinoid treatment of MCF-7 cells also suggests that compounds other than retinoids can be screened for their capacity to induce retinoid-inducible gene expression in the absence of retinoids or in cells lacking retinoid receptors. Such screening assays are performed as follows. A recombinant expression construct, prepared as described above and verified for inducibility by retinoids, is introduced by transfection into any mammalian cell line, whether sensitive or insensitive to retinoids, for example HT1080 cells (A.T.C.C. Accession No. CCL121) that are known to lack retinoic acid receptors. Stable transfectants are selected as described above using an appropriate amount of a selecting agent specific for the selectable marker encoded by the plasmid or vector, and selective agent-resistant cell lines are isolated thereby.

These cells are used in luciferase activity assays (using a Luciferase Assay System, Promega) as described above. These assays are performed on cells cultured in the presence or absence of increasing amounts of the compound to be tested for different periods of time, or cultured in compound-free media.

This assay is performed substantially as described above. Cells are plated at a density of 40,000 cells/well in 12 well plates in 1 mL of media containing penicillin/streptomycin, glutamine and 10% fetal calf serum (FCS). After attachment, cells are treated with increasing concentration of a compound to be tested, or left untreated for different periods of time. Cells are washed with PBS, collected in 300 μ L RLB, centrifuged briefly to remove debris, and then assayed as above using the Firefly Luciferase assay. Protein concentrations are determined to normalize the luciferase results, which are expressed as luciferase activity/ μ g protein. All assays are carried out in triplicate and displayed as a mean and standard deviation. Finally, cells are assayed in

parallel for growth inhibition by the tested compound using cell counting or measuring cell number after staining with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), methylene blue or other cell-specific stain

5 It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.